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the adverse immunological responses associated with gene delivery by these vectors.

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Transient Immunosuppression Allows Transgene Expression Following Readministration of Adeno-Associated Viral Vectors

WILLIAM C. MANNING, SHANGZHEN ZHOU, MARY PAT BLAND, JAIME A. ESCOBEDO, and VARAVANI DWARKI

ABSTRACT

Adeno-associated viral (AAV) vectors have much promise in gene therapy. Among the many properties that make AAV an ideal vector for gene therapy are its ability to infect both dividing and nondividing cells and the longevity of expression in tissues such as brain, skeletal muscle, and liver. However, like other viral vectors, readministration of vector is limited because of the host's immune response to viral components of the vector. Using class I, class II, and CD40 ligand (CD40L)-deficient mice, we demonstrate that neutralizing antibodies to the viral capsid proteins prevent transgene expression following readministration of rAAV vectors. Transient immunosuppression of mice by treatment with antibody to CD4 at the time of primary infection allowed transgene expression after readministration of rAAV vectors to animals. Transient immunosuppression with antibody to CD40L had only a modest effect on the efficacy of readministration. The ability to readminister virus was inversely correlated with both AAV capsid enzyme-linked immunosorbent assay titers and AAV neutralizing antibody titers. These studies demonstrate that readministration of rAAV can be accomplished by down regulating the anti-AAV immune response and suggest the use of repeated administration of rAAV as a viable form of therapy for the treatment of chronic diseases.

OVERVIEW SUMMARY

A problem common to all viral vectors in gene therapy is that the host can mount an immune response to the vector. This immune response prevents transgene expression following readministration of the viral vector. Our results show that the host's humoral immune response prevents intramuscular readministration of adeno-associated viral (AAV) vectors. By transiently immunosuppressing mice at the time of first administration with antibody to CD4, we show that readministration of rAAV vectors is possible.

INTRODUCTION

ADENO-ASSOCIATED VIRUS (AAV)-based vectors represent a promising gene delivery system (Muzyczka, 1992; Xiao *et al.*, 1993). AAV is a defective, nonpathogenic parvovirus and is incapable of autonomous replication and spread. One property of AAV that makes it an attractive vector for gene therapy is its ability to transduce both dividing and nondividing cells efficiently (Flotte *et al.*, 1994; Podsakoff *et al.*, 1994; Russell *et al.*, 1994; Halbert *et al.*, 1995). In addition, infection of many tissues, including skeletal muscle and brain, leads to persistent transgene expression (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Clark *et al.*, 1997; Fisher *et al.*, 1997). The vector system for generating rAAV is simple because the AAV terminal repeats are the only *cis*-acting elements that are necessary and sufficient for replication, packaging, and possibly integration (McLaughlin *et al.*, 1988; Samulski *et al.*, 1989). Recombinant AAV (rAAV) vectors lack virally encoded genes, and thus may avoid the potential problems associated with the host's immune response to viral proteins. rAAV preparations are stable and can be produced at high titers (>10^{12} particles/ml; Kessler *et al.*, 1996). There are reports on the utility of rAAV vectors in delivery to muscle (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Clark *et al.*, 1997; Fisher *et al.*, 1997; Herzog *et al.*, 1997), brain (Kaplitt *et al.*, 1994; McCown *et al.*, 1996), liver (Koeberl *et*

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al., 1997; Snyder *et al.*, 1997), lung (Flotte *et al.*, 1993; Afione *et al.*, 1996; Conrad *et al.*, 1996), and retina (Flannery *et al.*, 1997).

As the utility of rAAV-mediated gene delivery becomes more apparent, it is important to address some of the potential problems associated with this vector system. Like other viral vectors, the host can mount an immune response to the vector that would preclude its readministration for gene therapy. This response can include both an antibody and cellular response to the capsid of the vector particle itself and an immune response to the transgene and any viral proteins encoded by the vector genome (Yang *et al.*, 1994b; Dai *et al.*, 1995; Van Ginkel *et al.*, 1995). Because the rAAV genome does not encode any viral proteins, the primary host immune response may be against the capsid proteins of the vector particle. Neutralizing antibodies to the capsid could potentially prevent readministration of vector. To provide flexibility of repeat administration with rAAV vectors carrying the same or different transgenes, we have attempted to understand the immunological parameters that affect readministration and studied methods to overcome this problem.

Our experiments in mice demonstrate that rAAV cannot be readministered following intramuscular injection. By studying readministration in class I MHC, class II MHC, and CD40 ligand (CD40L)-deficient mice, we show that it is the host's humoral immune response to the vector that prevents effective second administration. Transient immunosuppression by anti-CD4 antibody treatment at the time of the initial injection resulted in transgene expression following second administration of rAAV. Treatment with anti-CD40L antibody was only modestly effective, and treatment with cyclosporin was not effective. Furthermore, the ability to readminister rAAV vector correlated with the ability to attenuate AAV capsid antibody and neutralizing antibody titers.

MATERIALS AND METHODS

Animals

Six-week-old female C57BL/6 mice were purchased from Charles River Labs (Wilmington, MA). C57BL/6 class I MHC-deficient and C57BL/6 class II MHC-deficient mice were purchased from Taconic Labs (Germantown, NY). The CD40 ligand-deficient mice and the B129 mice were purchased from Jackson Labs (Bar Harbor, ME). Prior to injection mice were anesthesized with a mixture of ketamine and xylazine. The rAAV was diluted in 0.9% saline and a final volume of 50 μl was injected into the tibialis anterior (TA) muscle.

For transient immunosuppression by anti-CD4 antibody, mice were injected with 100 μg of rat anti-mouse CD4 (clone GK1.5, Pharmingen, San Diego, CA) by intraperitoneal (i.p.) injection at days -3, 0, and $+3$ relative to the first injection of rAAV. For anti-CD40L treatment, mice received 100 μg of antibody (clone MR1, Pharmingen, San Diego, CA) by i.p. injection at days -3, 0, $+3$, and $+6$ relative to the first injection of rAAV. Mice treated with cyclosporin (Sandimmune, Sandoz) received i.p. injections of 10 mg/kg drug (diluted in corn oil) every 5 days from 1 week before the first injection of rAAV until the termination of the experiment.

Construction and preparation of recombinant AAV

pAAV-LacZ was constructed by cloning the LacZ expression cassette from pCMV-β (Clontech, Palo Alto, CA) containing the CMV promoter, intron, LacZ, and SV40 polyadenylation signal into pEMBL-AAV-ITR (Srivastava *et al.*, 1989). pAAV-Luc was constructed by cloning an expression cassette containing the CMV promoter/intron, luciferase (from pSP-*luc*+, Promega, Madison, WI), and the bovine growth hormone polyadenylation signal into pKm201. pKm201 is a derivative of pEMBL-AAV-ITR in which the ampicillin resistance gene was replaced with the gene for kanamycin resistance. pKSrep/cap was constructed by cloning the AAV-2 genome, without the ITRs (AAV-2 nucleotides 192–4,493) into pBluescript II KS+ (Stratagene, LaJolla, CA).

rAAV vector particles were produced by a modified transfection/infection protocol (Zhou *et al.*, 1994; Jordan *et al.*, 1996). Briefly, human embryonic kidney 293 cells, grown to 60% confluence in 15-cm dishes, were co-transfected with 12.5 μg of helper plasmid pKSrep/cap and 12.5 μg of vector plasmid pAAV-LacZ or pAAV-Luc using the calcium phosphate co-precipitation method. Eight hours after co-transfection, medium was replaced with Iscove's modified Dulbecco's medium (IMDM) + 10% FBS containing adenovirus type 5 *dl* 312 at a multiplicity of infection (moi) of 2. Seventy-two hours after infection, cells were harvested and resuspended in HEPES buffer (2.5 ml/dish) and lysed by three cycles of freezing and thawing. Cell debris was removed by centrifugation at 12,000 $\times$ *g* for 20 min. Packaged rAAV was purified from adenovirus by two rounds of cesium chloride equilibrium density gradient centrifugation. Residual adenoviral contamination was inactivated by heating at 56°C for 45 min. To estimate the total number of rAAV particles, the virus stock was treated with DNase I, and encapsidated DNA was extracted with phenol-chloroform and precipitated with ethanol. DNA dot blot analysis against a known standard was used to determine titer (Srivastava *et al.*, 1990). To assay for adenoviral contamination, 293 cells were infected with 10 μl of purified rAAV stock and followed for any signs of cytopathic effect. All stocks were negative for adenoviral contamination (level of detection greater than or equal to 100 pfu/ml). To assay for wild-type AAV, 293 cells were co-infected with serial dilutions of rAAV stocks and adenovirus *dl*312 at a moi of 2. Three days later, the cells were harvested, lysed by three cycles of freezing/thawing, and centrifuged to remove cell debris. The supernatant was heat inactivated (56°C for 10 min) and fresh plates of 293 cells (6 $\times$ 10^6) were infected in the presence of adenovirus *dl*312 at a moi of 2. Forty-eight hours after infection, low-molecular-weight DNA was isolated (Hirt, 1967), subjected to agarose gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized with a biotinylated oligonucleotide probe specific for the AAV capsid region. The wild-type AAV titer was defined as the highest dilution of rAAV vector stock, demonstrating a positive hybridization signal. Our rAAV preparations contained approximately one wild-type AAV genome per 10^6 rAAV genomes.

Luciferase assay

Muscles were frozen immediately upon harvest in liquid nitrogen. To prepare a lysate, frozen muscles were ground in a prechilled mortar and pestle, transferred to a 1.5-ml microfuge

tube, and resuspended in 500 μl of 1 × Reporter Lysis Buffer (Promega, Madison, WI). The tubes were vortexed for 15 min at room temperature and then freeze/thawed three times. Lysates were cleared by centrifuging at maximum speed in a microfuge for 10 min and then stored at −80°C until assayed. Luciferase assays were performed using the manufacturer's protocol (Promega, Madison, WI) and read on a Dynatech ML3000 (Dynatech, Chantilly, VA) plate luminometer. The protein concentration of each lysates was assayed by BCA protein assay (Pierce, Rockford, IL), and luciferase activities were expressed as picograms of luciferase per milligram of protein.

LacZ staining of muscle

Cryosections (8 μm) were fixed for 5 min at room temperature in 10 m*M* phosphate-buffered saline (PBS) containing 1% paraformaldehyde. The fixed sections were stained with X-Gal solution (PBS containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 1 m*M* $MgCl_2$, 5 m*M* $K_3Fe(CN)_6$, and 5 m*M* $K_4Fe(CN)_6$ for 16 hr at 37°C. Sections were counterstained with Nuclear Fast Red.

AAV capsid ELISA and neutralizing antibody assay

To perform the AAV capsid ELISA, microtiter plates were coated overnight at 4°C with 10^9 rAAV-LacZ particles/well in PBS. The following day, the plates were washed and then blocked for 30 min at 37°C with PBS containing 1% goat serum and 0.3% Tween 20. Serial three-fold dilutions of sample and control sera were loaded onto the plate starting at 1:75 (control sera was from mice that had received multiple injections of AAV). The microtiter plate was then incubated for 1 h at 37°C. Plates were washed and incubated at 37°C for 30 min with goat anti-mouse Ig-HRP at 1:2,000 (Dako, Carpenteria, CA). *o*-Phenylenediamine substrate was used to develop the plates. Plates were read at 492 nm with a cut-off of 0.2 OD. To perform the AAV neutralizing antibody assay, 293 cells were plated at 3 × 10^4 cells/well in a 96-well microtiter plate. The following day, prebleed, positive control, and sample sera were inactivated at 56°C for 30 min. Three-fold serial dilutions of sera in IMDM without fetal calf serum (FCS; Biowhittaker, MD) were then incubated with 10^8 particles of rAAV-Luc at 37°C for 1 hr. The medium was removed from the 293 cells, and diluted sera plus virus were added and incubated for 1 hr at 37°C. After this incubation, the plates were washed and fresh IMDM containing 10% FCS was added. Twenty-four hours later, cells were rinsed with PBS and lysed in Reporter Lysis Buffer (Promega, Madison, WI). Cells were then harvested and assayed for luciferase activity. The AAV neutralizing antibody titer was defined as the dilution of serum required to see 50% of the luciferase activity in 293 cells infected with rAAV-Luc preincubated with negative control serum.

RESULTS

The host humoral immune response is responsible for the lack of transgene expression following readministration of rAAV vectors

Previous experiments in our laboratory had demonstrated that after a single intramuscular (i.m.) injection of a rAAV vector, transgene expression from a second vector injection was not possible in BALB/c or C57BL/6 mice (unpublished observations). To determine which arm of the host immune response was responsible for the inability to readminister rAAV vectors, experiments were carried out in class I, class II, and CD40L-deficient mice. Class I deficient mice do not develop a normal population of CD 8^+ T cells and are unable to mount cellular immune responses (Zijlstra *et al.*, 1990). Class II-deficient mice are negative for CD 4^+ T cells and are defective in humoral immune responses (Grusby *et al.*, 1991). On day 0, groups of 5 class I-deficient, class II-deficient, or C57BL/6 mice were injected with 1 × 10^{10} particles of rAAV-LacZ in the right tibialis anterior (TA) muscle. At 4 weeks following the first injection, serum was collected, and the mice were reinjected with either 1 × 10^{10} particles rAAV-LacZ (three animals) or 1 × 10^{10} particles rAAV-Luc (two animals) in the left TA. At 6 weeks after the first injection, the mice were sacrificed, and muscles were collected for either LacZ staining or luciferase assay. As shown in Table 1, no LacZ staining and only relatively low levels of luciferase expression were found in C57BL/6 mice previously injected with rAAV-LacZ. High levels of luciferase expression and strong LacZ staining were found in the left TA muscles of Class II-deficient mice, and intermediate levels were found in the left TA muscles of class I-deficient mice. AAV capsid and neutralizing antibody titers were determined at the time of second injection (4 weeks), and are shown in Fig. 1. The control C57BL/6 mice had high capsid antibody and neutralizing antibody titers whereas the class II-deficient mice did not have detectable AAV capsid or neutralizing antibody titers. Surprisingly the antibody titers in the class I-deficient mice were lower than that found in the C57BL/6 mice and resulted in an intermediate level of luciferase or LacZ gene expression following second rAAV administration. The observation that second administration of both rAAV-LacZ and rAAV-Luc was inefficient in the C57BL/6 mice indicated that an immune response to the transgene was not responsible for the failure of readministration. Readministration experiments using rAAV-LacZ for the first and second injection were also performed in BALB/c mice with similar results (data not shown).

CD40L deficient mice can not mount normal humoral immune responses (Xu *et al.*, 1994; Whitmire *et al.*, 1996). An experiment similar to that described for the class I- and class II-deficient mice was performed in CD40L-deficient mice, except that in all mice the second injection was rAAV-Luc. As shown in Table 2, effective second administration of rAAV was not possible in the wild-type control mice (B129), but possible in the CD40L-deficient mice. As was the case in the previous experiment, the ability to readminister vector correlated with capsid and neutralizing antibody titers. It is interesting to note that rAAV-Luc transduction of skeletal muscle in the B129 strain (the background strain of the CD40 ligand-deficient mice) was, in general, less efficient than that observed in C57BL/6 mice (Tables 1 and 3).

The effect of first injection dose on efficacy of transgene expression following readministration

To determine whether the dose of the first injection affected the efficacy of the second injection, groups of 5 C57BL/6 mice

TABLE 1. rAAV-MEDIATED TRANSGENE EXPRESSION FOLLOWING READMINISTRATION IN CLASS I- AND CLASS II-DEFICIENT MICE

Strain and animal	*Treatment*[a]		*LacZ staining*[b] *(2 weeks post second)*		*Luciferase activity*[c] *(2 weeks post second)*
	First injection (right)	*Second injection (left)*	*Right*	*Left*	*Left*
C57BL/6					
1	LacZ	LacZ	++	—	N.A.
2	LacZ	LacZ	++	—	N.A.
3	LacZ	LacZ	++	—	N.A.
4	LacZ	Luc	++++	N.A.[d]	158
5	LacZ	Luc	++	N.A.	50
CLASS I neg					
1	LacZ	LacZ	++++	++	N.A.
2	LacZ	LacZ	++++	+	N.A.
3	LacZ	LacZ	+++	+	N.A.
4	LacZ	Luc	+++	N.A.	581
5	LacZ	Luc	+++	N.A.	825
CLASS II neg					
1	LacZ	LacZ	++++	++++	N.A.
2	LacZ	LacZ	++	++++	N.A.
3	LacZ	LacZ	+++	+++	N.A.
4	LacZ	Luc	++++	N.A.	2,744
5	LacZ	Luc	+++	N.A.	8,450

[a]All injections contained 1×10^{10} particles rAAV.
[b]—, no staining; +, 1–10%; ++, 10–50%; +++, 50-90%; ++++, >90%.
[c]pg luciferase per mg of protein.
[d]Not applicable.

were injected with escalating doses of rAAV-LacZ in the right TA, and then injected with 1×10^{10} particles rAAV-Luc in the left TA 4 weeks after the first injection. As shown in Fig. 2A, the groups that received the lower doses of virus (10^4, 10^6) expressed luciferase following readministration as efficiently as the naive controls. These same groups of mice also did not develop neutralizing antibodies to AAV, indicating that the amount of capsid antigen in these doses may have been too low to elicit an immune response (Fig. 2B). In fact, the right TA muscles of these low-dose groups of mice were also negative for LacZ staining at the time of sacrifice (data not shown). The group that received 10^8 particles of rAAV-LacZ mounted a relatively weak antibody response to AAV. This lower antibody response led to an intermediate level of luciferase expression from the second injection. In this group, there was a reverse correlation between the levels of neutralizing antibody and luciferase expression. The group receiving 10^{10} particles demonstrated a robust antibody response to AAV and second administration did not result in luciferase expression.

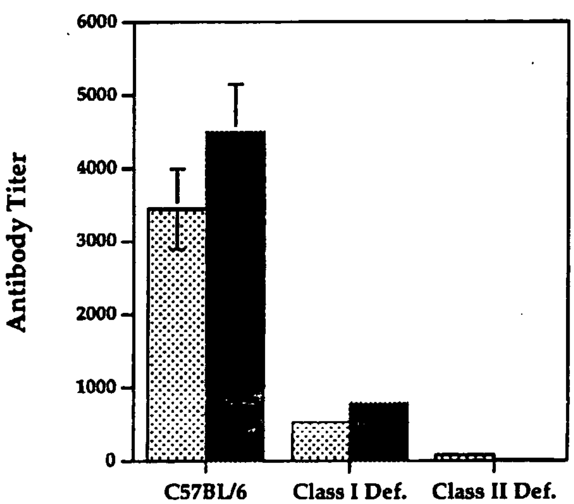

FIG. 1. AAV capsid and neutralizing antibody titers in C57BL/6, class I-deficient, and class II-deficient mice following i.m. injection of rAAV. AAV capsid antibody titer (dotted bars) and AAV neutralizing antibody titer (solid bars) were determined 4 weeks after i.m. injection of rAAV-LacZ. Titers represent the mean ± SEM (n = 5).

Transient immunosuppression with anti-CD4 antibody resulted in transgene expression following readministration of rAAV

On the basis of the results of the previous experiments, which indicated that antibody to vector blocked readministration, we attempted to attenuate the host's antibody response to rAAV by transient immunosuppression. As shown in Table 3, readministration of rAAV was achieved in 60% of the mice treated with anti-CD4 antibody at the time of first injection. On average, luciferase expression in the CD4 antibody-treated mice was approximately 40% the level observed in naive mice. Individual anti-CD4 treated animals, however, demonstrated levels of luciferase expression comparable to the naive control group. To ensure that the $CD4^+$ T cell depletion was successful, blood

TABLE 2. GENE EXPRESSION FOLLOWING READMINISTRATION OF rAAV IN CD 40 LIGAND-DEFICIENT MICE

Strain and animal	*Treatment*[a]		*Luciferase activity*[b] *(2 weeks post second injection)*	*Anti-AAV titer (at time of second injection)*	*AAV neutralizing titer (at time of second injection)*
	First injection	*Second injection*			
B129	Nothing	rAAv-Luc			
1			14	N.D.	N.D.
2			17	N.D.	N.D.
3			80	N.D.	N.D.
4			38	N.D.	N.D.
5			83	N.D.	N.D.
			46 ± 15		
B129	rAAV-LacZ	rAAV-Luc			
1			0	2,515	10,000
2			0	5,179	20,000
3			0	502	1,500
4			0	316	1,500
5			0	3,097	7,500
			0		
CD40L neg.	Nothing	rAAV-Luc			
1			267	N.D.	N.D.
2			15	N.D.	N.D.
3			105	N.D.	N.D.
4			233	N.D.	N.D.
5			159	N.D.	N.D.
			156 ± 45		
CD40 L neg.	rAAV-LacZ	rAAV-Luc			
1			200	<75	<75
2			533	<75	<75
3			25	<75	<75
4			19	<75	<75
5			20	<75	<75
			159 ± 100		

[a]All injections contained 1×10^{10} particles rAAV.
[b]pg luciferase per mg of protein; average ± SEM is also shown.

was collected from all anti-CD4 antibody-treated mice on day +4 and analyzed by flow cytometry. All mice showed greater than 99% reduction in the number of $CD3^+$ $CD4^+$ T cells and had normal numbers of $CD3^+$ $CD8^+$ T cells (data not shown). As was seen in the previous experiments, there was an inverse correlation between luciferase expression and AAV antibody titer. A similar experiment was performed using anti-CD40L antibody treatment. In this experiment, 2 of 3 animals achieved low levels (approximately 4% of naive controls) of luciferase expression following the second injection, indicating that anti-CD40L antibody treatment alone is probably not optimal for readministration. The AAV capsid antibody titers in these 2 animals were also higher than in the anti-CD4 antibody-treated animals. The anti-CD4 and anti-CD40L antibody-treated groups demonstrated AAV neutralizing antibody titers 2 weeks after the second injection, indicating that the immunosuppression was transient (neutralizing titers 2 weeks post second injection in the CD4 and CD40L antibody-treated animals were not statistically different from the control group when analyzed by Fisher's paired least-squares difference test for pairwise comparisons). Both the anti-CD4 (5 animals) and anti-CD40L (3 animals) antibody experiments were repeated with similar results (data not shown). Treatment with cyclosporin had no effect on the ability to readminister rAAV.

DISCUSSION

In summary, we have shown by readministration experiments in class II-deficient mice that it is primarily the host's humoral immune response to the rAAV capsid that prevents efficient transgene expression after i.m. readministration of rAAV vectors. We have also shown that transient immunosuppression with anti-CD4 antibody, and to a lesser extent with anti-CD40L antibody, allowed transgene expression following readministration of vector. Furthermore, the ability to readminister vector correlated with a reduction in AAV capsid and neutralizing antibody titers.

Gene delivery experiments performed with adenoviral vectors demonstrate that the intracellularly expressed viral antigens elicit strong cellular immune responses that lead to elimination of transduced cells (Yang *et al.*, 1994a). In contrast to adenoviral vectors, AAV vectors have the potential to avoid this problem because they do not express any viral genes. rAAV vectors carry the capsid proteins of the virus which are responsible for efficient entry and possibly transport of the packaged DNA to the nucleus. It is not clear whether the capsid, when delivered as protein antigen, could elicit a strong cellular immune responses leading to destruction of the target cells. It is possible to speculate that because the capsid proteins of

TABLE 3. EFFECT OF TRANSIENT IMMUNOSUPPRESSION ON TRANSGENE EXPRESSION IN C57BL/6 MICE FOLLOWING READMINISTRATION OF rAAV

Treatment[a] and animal: First injection	Second injection	Luciferase activity[b] (2 weeks post second injection)	Anti-AAV titer (at time of second injection)	AAV neutralizing titer (at time of second injection)	AAV neutralizing titer[c] (2 weeks post second injection)
Nothing	rAAv-Luc				
1		107	N.D.	N.D.	4,000
2		486	N.D.	N.D.	2,000
3		605	N.D.	N.D.	450
4		220	N.D.	N.D.	2,500
5		4,671	N.D.	N.D.	800
6		800	N.D.	N.D.	5,000
7		1,188	N.D.	N.D.	2,500
		1,154 ± 602			2,464 ± 629
rAAV-LacZ	rAAV-Luc				
1		28	3,908	4,000	5,000
2		6	9,326	2,000	6,500
3		14	4,564	1,000	3,000
4		5	7,014	2,500	6,500
5		6	1,597	1,250	17,500
		12 ± 4			7,700 ± 2,532
rAAV-LacZ[d] (α-CD 4 treated)	rAAV-Luc				
1		2	124	800	7,000
2		2	43	<15	<15
3		575	121	70	9,000
4		317	47	<15	4,000
5		3	2,932	1,500	15,000
6		356	<15	<15	1,000
7		920	<15	<15	2,000
8		772	60	<15	3,000
9		1,064	<15	<15	2,000
10		19	408	550	6,500
		403 ± 129			4,900 ± 1,439
rAAV-LacZ (α-CD 40 ligand treated)	rAAV-Luc				
1		94	489	500	8,000
2		31	477	900	4,000
3		4	2,035	1,000	100,000
		43 ± 27			37,333 ± 31,354
rAAV-LacZ (cyclosporin treated)	rAAV-Luc				
1		0	10,911	8,000	N.D.
2		0	2,497	5,500	N.D.
3		0	10,394	20,000	N.D.
4		0	11,827	10,000	N.D.
5		0	6,777	10,000	N.D.
		0			

[a]All injections were 1×10^{10} particles rAAV unless otherwise noted.
[b]pg luciferase per mg of protein; average ± SEM is also shown.
[c]Average titer ± SEM is also shown.
[d]Animals 1–7 received 1×10^{10} rAAV-LacZ in both the right and left muscle at time of first injection.

AAV are not expressed *de novo* in the cell, they may not be presented efficiently on class I molecules. Class I presentation of capsid peptides would also be transient. The reports of long-term transgene expression following i.m. delivery of rAAV vectors also support this hypothesis.

Our experiments with class I and class II MHC-deficient mice provide evidence for the importance of the humoral immune response in readministration. Class II-deficient mice could be efficiently reinfected with rAAV vectors in contrast to either the class I or control mice. In comparison to control immune-competent mice, class I-deficient mice were partially susceptible to readministration. The partial success of readministration in the class I-deficient mice may be due to their reduced AAV capsid and neutralizing antibody titers. A similar deficiency in the neutralizing antibody response to an adenoviral vector has also been reported in class I-deficient mice

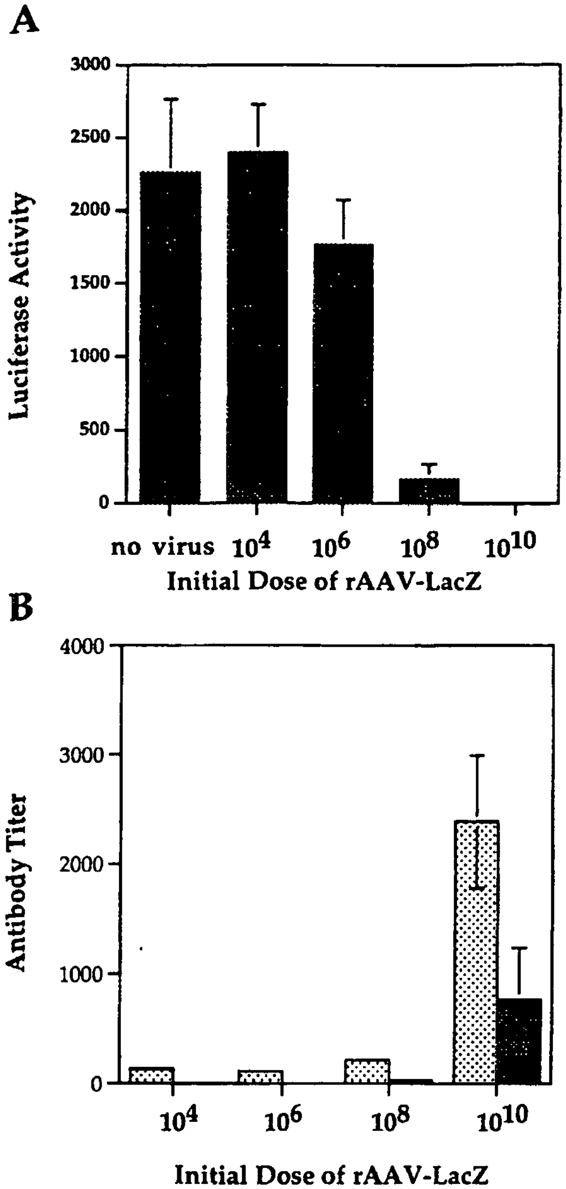

FIG. 2. Effect of the dose of primary rAAV injection on the efficacy of gene expression following second administration of rAAV. A. Luciferase activity (pg luciferase per mg of protein) in the left TA muscle 2 weeks after the second injection with rAAV-Luc. The amount of rAAV-LacZ injected in the right TA in the first injection is indicated on the x axis. Values represent the mean ± SEM (n = 5). B. Effect of primary injection dose on antibody response to rAAV. AAV capsid antibody titer (dotted bars) and AAV neutralizing antibody titer (solid bars) were determined 4 weeks after i.m. injection of rAAV-LacZ. The dose of rAAV-LacZ is indicated on the x axis. Titers represent the mean ± SEM (n = 5).

(Yang *et al.*, 1996). The correlation between the strength of the AAV capsid antibody titer and the ability to readminister is evident from these experiments and clearly demonstrates that the humoral arm of the immune system plays a key role in preventing readministration.

To establish further the role of the humoral immune response in readministration, we performed experiments in CD40L-deficient mice. CD40L is expressed on activated $CD4^+$ T cells and is critical for their ability to provide help to B cells (Durie *et al.*, 1994; Xu *et al.*, 1994; Yang and Wilson, 1996). Thus, the CD40L-deficient mice should mimic the responses seen in the class II MHC-deficient mice. Our results demonstrated that as in the class II-deficient mice, the CD40L-deficient mice could be effectively administered a second dose of rAAV. It is interesting to note that the rAAV-mediated transgene expression levels in this strain of mice (B129 background) were much lower than in the C57BL/6 background. This illustrates the fact that mouse strain haplotype can influence the expression levels mediated by rAAV vectors.

Results from the class I-deficient mice indicated that antibody titer influenced the ability to administer the second dose of virus. In a dose–response experiment, we found that a dose of at least 10^8 rAAV particles was needed to generate an antibody response to AAV capsids that could block readministration. Thus, our experiments demonstrate the relationship between the appearance of neutralizing antibody and the efficacy of readministration.

On the basis of these results, we attempted several strategies to block the humoral immune response and test the feasibility of readministration. Our rationale for choosing the anti-CD4 antibody and anti-CD40L antibody was to block the humoral arm of the immune system. Cyclosporin was chosen as a more general immunosuppressive agent. We chose a transient immunosuppresive regimen because this method has practical utility for future gene therapy experiments in humans. Our results demonstrated that blocking the humoral response during the primary administration of vector by treatment with antibody to CD4 allowed effective gene transfer following second administration. In this experiment, we could demonstrate effective gene expression following second injection in 60% of the animals. It is important to note that animals that responded poorly to the second administration had, in general, higher neutralizing antibody titers (Table 3). The variability in luciferase expression levels (from 107 to 4,671 pg luciferase/mg protein) observed in the animals that received anti-CD4 antibody is probably due to the variability inherent in muscle based delivery.

The effect of CD40L antibody treatment on readministration was not as dramatic as that seen with anti-CD4 antibody treatment. It is not clear if the anti-CD40L antibody treatment regimen was optimal in blocking the humoral response. A recent report has shown that transient immunomodulation with a combination of anti-CD40L antibody and CTLA4Ig enhanced persistence and secondary adenovirus-mediated gene transfer in the mouse liver (Kay *et al.*, 1997). They also demonstrated that co-administration was more effective than using either agent alone. Thus, it is possible that more than one molecule needs to be targeted in the pathway to ensure sufficient blocking of the humoral immune response. Our results with cyclosporin demonstrated that this molecule, though useful in the prevention of graft rejection, is not useful in blocking the antibody response elicited by rAAV administration.

We have shown that it is possible to readminister rAAV vectors by following regimens that can attenuate the host's antibody response against the rAAV capsid. Our data also demon-

strate that transient immunosuppression is sufficient to accomplish this goal. It is also important to point out that because the rAAV vectors do not carry virally encoded genes, cellular responses to the capsid may not pose a problem in elimination of target cells. Thus, if the transgene being delivered is a self-protein, the issue that needs to be addressed is the prevention of the host's humoral immune response against the rAAV capsid. Because many individuals are already seropositive for AAV (Blacklow *et al.*, 1968), it will be of interest to determine the levels of neutralizing antibody necessary for prevention of rAAV administration. Our dosing experiment suggests that relatively low levels of neutralizing antibody in mice can have an effect on readministration.

Data from several groups also corroborate our finding regarding the lack of readministration following i.m. administration of rAAV vectors (Xiao *et al.*, 1996; Clark *et al.*, 1997). One group reported that it was possible to readminister rAAV after a primary intramuscular injection even though neutralizing antibodies were present (Fisher *et al.*, 1997). The dose of rAAV-LacZ they used (10^9 vector genomes) was lower than the dose we used in our rAAV-LacZ experiments (Table 1). It is possible that by using a lower primary injection dose, the host's lower AAV neutralizing antibody response would allow some level of gene expression following second administration. Our dosing experiments clearly showed a dose dependence on AAV antibody titer and the ability to readminister vector effectively.

Repeated administration of the same rAAV vector or subsequent administration of different rAAV vectors may be necessary in the treatment of certain diseases. For these situations, it will be crucial to develop effective methods of transient immunomodulation if gene therapy is to be successful. Our results demonstrate that an understanding of the immune components responsible for the block in second administration of rAAV can lead to the development of effective strategies to overcome this problem.

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